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Telomerase as an Androgen Receptor-Regulated Target in
Selenium Chemoprevention of Prostate Cancer

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14. ABSTRACT The present is to investigate telomerase as a potential target of AR signaling suppression by selenium. We found that combination of selenium and bicalutamide produced a robust down-regulation of androgen receptor (AR) signaling and telomerase activity. Furthermore, apoptosis induction by the two agents is more significant compared to single treatment. Our findings thus indicate that selenium in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of androgen deprivation therapy. We also found that wild-type and mutant AR responses differentially to androgen under normoxia condition. However, in hypoxia condition, androgen up-regulates hTERT expression in both wild-type AR expressing cells and mutant AR expressing cells. Selenium is able to inhibit androgen-induced hTERT expression under hypoxia condition.					
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A. INTRODUCTION:

This project was designed to investigate telomerase as a potential target of androgen receptor (AR) signaling suppression by selenium. In the first annual report, we described that over-expression of hTERT attenuates the apoptosis inducing activities of selenium, supporting an important role of hTERT in selenium action in prostate cancer cells. Our data also provided an array of evidence supporting selenium in combination with an anti-androgen as a potential new modality for prostate cancer treatment. More importantly, we indentified hTERT/telomerase as an important AR target mediating the bicalutamide/selenium effect. We also found that selenium can induce DNA damage response in LNCaP cells. In addition, our data showed that androgen-stimulated AR signaling induces the expression of hTERT through up-regulating hTERT promoter activity. During this second year of funding period, we continued to investigate the mechanism by which hTERT/telomerase suppression mediates the anti-cancer effect of selenium, and further examined the combinatorial effect of bicalutamide and selenium on telomerase activity and cell growth. As approved by the DOD Prostate Cancer Research Program, we continued our study on the transcriptional regulation of hTERT by AR signaling, but shifted our research focus to the differential response of hTERT to androgen under normoxia vs. hypoxia condition.

B. BODY:

Aim 1: To assess the cellular mechanism by which hTERT/telomerase down-regulation mediates the anti-cancer effect of methylseleninic acid (MSA)

Experiment 1. Combined bicalutamide and MSA treatment has enhanced induction of apoptosis and inhibition of hTERT expression and telomerase activity.

Androgen deprivation therapy (ADT) is the mainstay treatment for advanced prostate cancer. It targets the action of androgen receptor (AR) by reducing androgen level and/or by the administration of anti-androgen that competes with androgens for binding to AR. Albeit effective in extending survival, ADT is associated with dose-limiting toxicity and the development of castration-resistant prostate cancer (CRPC) after prolonged use. Since CRPC is generally lethal and incurable, developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. Continuous AR signaling constitutes one major mechanism underlying the development of CRPC. Our finding showed that MSA, an agent that effectively reduces AR abundance, could enhance the cancer-killing efficacy of the anti-androgen bicalutamide in both androgen-dependent and castration-resistant prostate cancer cells, thus indicate that MSA in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of ADT.

In our first annual report, we investigated the combinatorial effect of anti-androgen and MSA on cell growth, AR signaling and hTERT/telomerase. The purpose of this study is to determine the potential of using MSA to increase the cancer-killing efficacy of anti-androgen in both androgen-dependent and castration-resistant prostate cancer cells. We have shown that the combination treatment has greater inhibition on AR transcriptional activity, hTERT mRNA expression and cell growth. In this second year period, we further examined the combinatorial effect on AR protein expression, telomerase activity and cell growth.

LNCaP and LN3 cells were treated with bicalutamide and/or MSA for 48 hr, and apoptosis was analyzed by using the Cell Death ELISA assay. As shown in Fig. 1, in both cell models,

bicalutamide and MSA in combination led to a more striking increase of apoptosis than either agent alone.

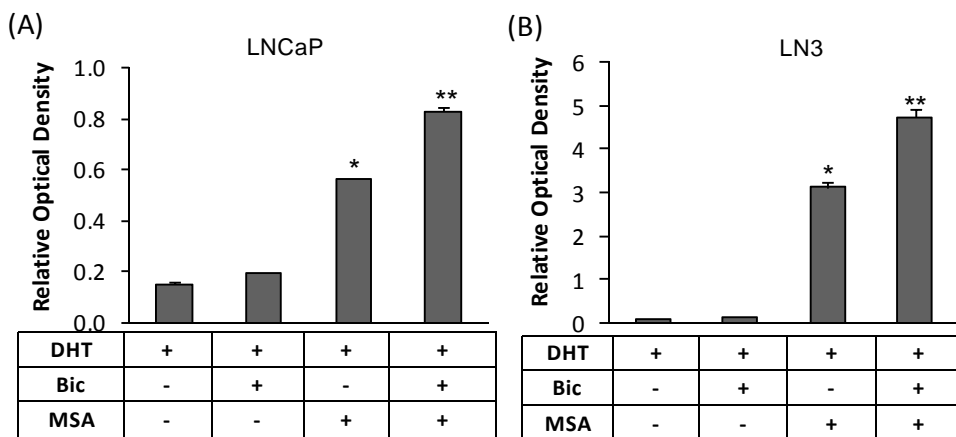


Figure 1. Bicalutamide (Bic) and MSA induction of apoptosis. Induction of apoptosis as determined by the Cell Death ELISA assay in LNCaP cells treated with 5 μ M Bic, 2.5 μ M MSA, or Bic and MSA in combination (A), in LN3 cells treated with 10 μ M Bic, 5 μ M MSA, or Bic and MSA in combination (B) for 48 hr. The data obtained by *ELISA* were *normalized* by SRB results for cell content. *, $P < 0.01$ compared to DHT-treated sample. **, $P < 0.01$ compared to Bic- or MSA-treated sample.

Conversely, the combination efficacy was not observed in the AR-negative DU-145 cell line (Fig. 2). In concordance with the previously-reported AR-independent activity of MSA in prostate cancer cells (1-5), MSA treatment of DU-145 cells resulted in a marked induction of apoptosis. However, the combination with bicalutamide was not able to lead to further induction of apoptosis. The above data highly suggest that the suppression of hTERT by bicalutamide and MSA combination treatment is mainly through AR signaling pathway.

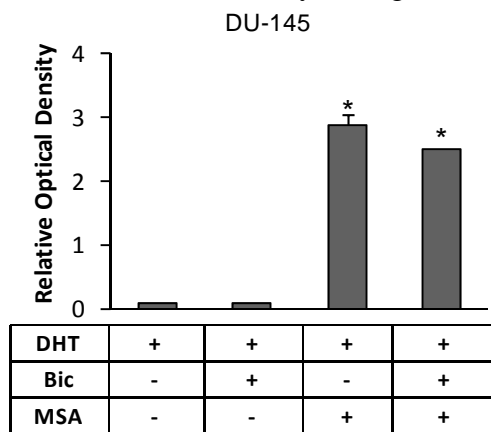


Figure 2. Bicalutamide (Bic) and MSA induction of apoptosis. DU-145 cells were treated with 10 μ M Bic, 2.5 μ M MSA, or Bic and MSA in combination for 48 hr. The data obtained by *ELISA* were *normalized* by SRB results for cell content. *, $P < 0.01$ compared to DHT-treated sample.

We then looked at the effect of bicalutamide and MSA combination on suppression of AR protein expression. Western blot result shows that in both LNCaP and LN3 cells, combination treatment could reduce more AR protein compared with single agents (Fig. 3). This is consistent with the repression of AR trans-activation (as shown in first annual report).

Next we proceeded to evaluate the effect of bicalutamide in combination with MSA on telomerase activity by using TRAP assay. As shown in Fig. 4A, telomerase activity in LNCaP cells was markedly induced by dihydrotestosterone (DHT). Bicalutamide or MSA alone produced minimal inhibition of DHT-induced telomerase activity. The two drugs in combination, however, almost completely blocked DHT induction of telomerase activity. The combination effect on telomerase activity was also manifested in LN3 cells (Fig. 4B). Thus, the telomerase

activity data were qualitatively similar to the hTERT mRNA result (as shown in first annual report).

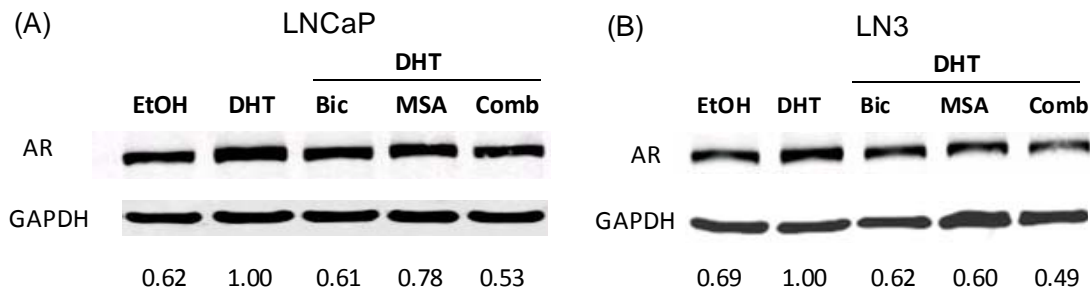


Figure 3. Bicalutamide (Bic) and MSA effect on AR protein expression. AR protein level was determined by using Western Blot in LNCaP cells treated with 5 μ M Bic, 2.5 μ M MSA, or Bic and MSA in combination (A), in LN3 cells treated with 10 μ M Bic, 5 μ M MSA, or Bic and MSA in combination (B) for 6 hr. The quantization was normalized by GAPDH.

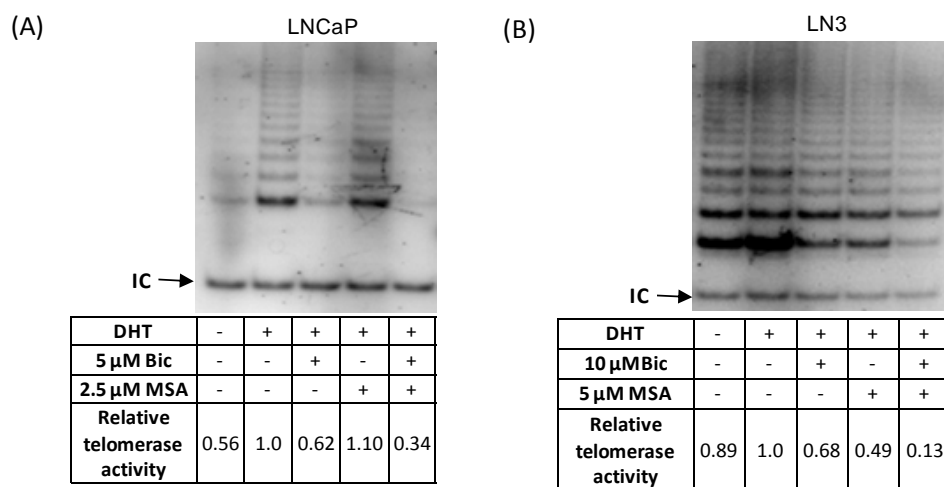


Figure 4. Bicalutamide (Bic) and MSA downregulation of telomerase activity. LNCaP cells were treated with 5 μ M Bic and/or 2.5 μ M MSA, and LN3 cells with 10 μ M Bic and/or 5 μ M MSA. The activity of telomerase in LNCaP (A) and LN3 (B) cells were analyzed by the TRAP assay. IC represents internal control. The numbers are normalized telomerase activities relative to the DHT-treated sample.

Experiment 2. To investigate the mechanism by which hTERT/telomerase suppression mediates the anti-cancer effect of MSA.

Our previous studies showed that 10 μ M MSA induces a marked growth inhibition of prostate cancer cells at 48 hr. Telomerase suppression by MSA is unlikely to result in appreciable telomere shortening within such a short period of time. As expected we did not observed any change of telomere length up to 72-hour MSA treatment (as shown in Fig.8 in first annual report). Therefore, a mechanism independent of telomere shortening, such as telomere capping status, should be considered. Uncapped telomeres have been reported to trigger a rapid DNA damage response and lead to cell cycle arrest and/or apoptosis (6). In our first annual report, we showed that MSA could induce DNA damage marker, indicating there is DNA damage response going on under MSA treatment. However, we had some technique problems to stain the MSA-treated cells. We then modified our protocols, LNCaP cells were treated with or

without 10 μ M MSA for 24 hr, and immunofluorescent staining of 53BP1 was performed. As shown in Fig. 5, MSA induced more DNA damage foci than in control cells. To delineate these foci are located in telomere, we tried to co-stain 53BP1 with TRF1, which is a telomere associated protein used to indicating the location of telomere. We still need to work on the double staining method to show solid data.

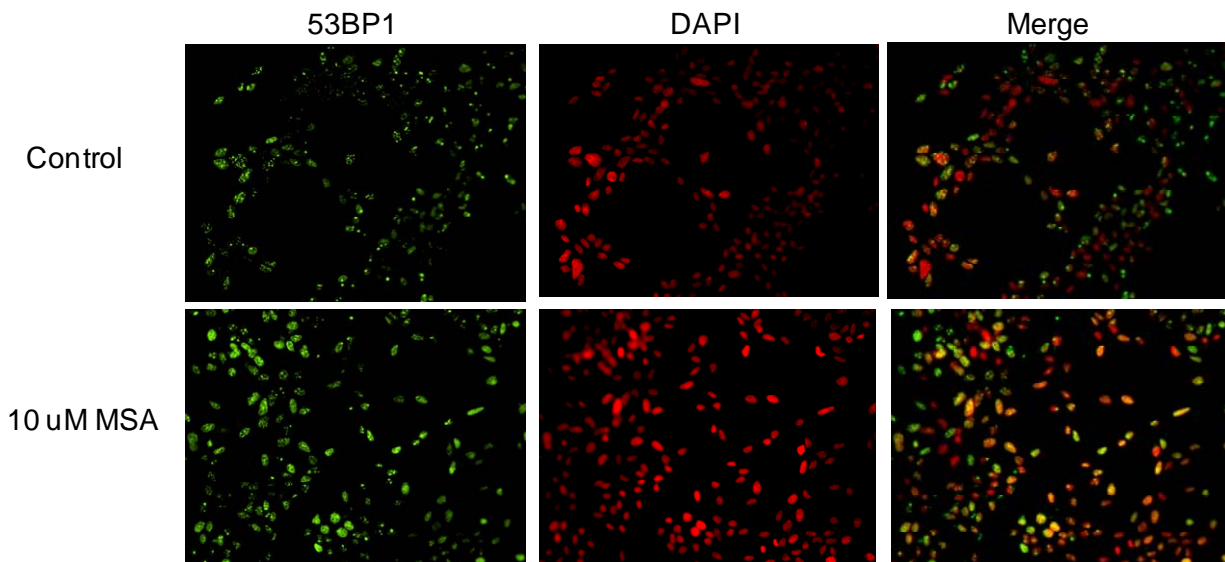


Figure 5. MSA induces DNA damage response in LNCaP cells. Immunofluorescentstaining of 53BP1 in LNCaP cells treated with or without 10 μ M MSA for 24 hr.

Aim 2: To study why do LNCaP and LAPC-4 cells respond differentially to DHT treatment.

In our first annual report we showed an interesting phenomenon that wild-type and mutant AR have different function in term of regulation of hTERT expression. Since the mechanism of hTERT regulation by AR is not well studied, it would be more important to address this issue. Therefore, we requested a change on our previous **Aim 2**. In this second year period, we continued our study of the mechanism by which AR up-regulates hTERT expression by studying the association of AR with androgen-responsive element (ARE) in hTERT promoter region, and also studied the androgen effect on hTERT expression under both normoxia and hypoxia conditions to investigate why wild-type and mutant AR respond differentially to androgen treatment regarding hTERT expression.

Experiment 1. Reduction of AR occupancy of the hTERT promoter by MSA.

In order to demonstrate that suppressed hTERT promoter activity is attributable to reduced AR occupancy of the hTERT promoter as a consequence of MSA downregulation of AR, we performed the Chromatin Immunoprecipitation (ChIP) assay on the hTERT promoter region that was reported to associate with AR (7). Fig. 6A shows the result of PCR amplification of DNA co-immunoprecipitated with either an AR antibody or IgG using primers specific for the hTERT promoter region. The specificity of the ChIP assay is evident from the difference in band intensity between the AR-antibody-immunoprecipitated samples and the IgG-immunoprecipitated samples. In concordance with a previous report (7), AR is only weakly associated with the hTERT promoter in the absence of androgen in LNCaP cells. DHT significantly increased AR occupancy of the promoter, and the increase was blocked by 10 μ M MSA. We then conducted quantitative real-time PCR (qRT-PCR) of the co-immunoprecipitated DNA to quantify the change in AR occupancy of hTERT promoter in response to different

treatments. As shown in Fig. 6B, DHT treatment induced a ~6-fold increase of AR occupancy of the promoter, and the increase was completely suppressed by MSA.

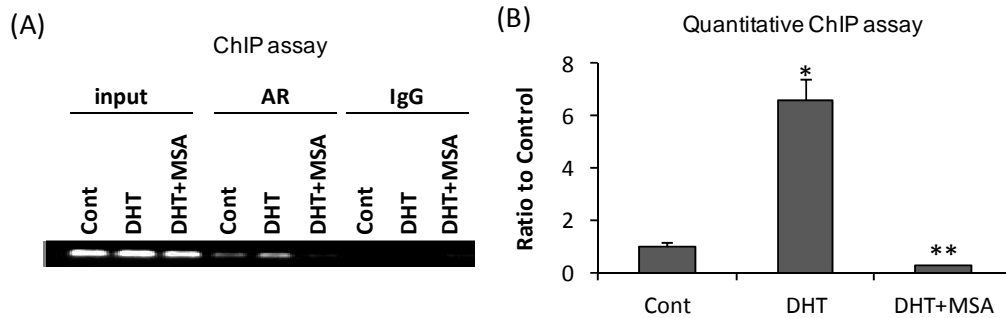


Figure 6. MSA reduction of AR occupancy of hTERT promoter as determined by ChIP assay (A) or quantitative ChIP assay (B). The assay was conducted at 16 hr after treatment with 1 nM DHT and/or 10 μ M MSA. Input, total input chromatin for each treatment sample. AR, DNA co-immunoprecipitated with an AR antibody. IgG, DNA co-immunoprecipitated with an IgG. *, $P < 0.01$, significantly different from ethanol-treated control cells cultured in charcoal-stripped fetal bovine serum. **, $P < 0.01$, significantly different from DHT-treated sample.

Experiment 2. Androgen signaling regulation of hTERT expression under normoxia and hypoxia conditions.

LNCaP cells were cultured with androgen-deprived medium under both normoxia and hypoxia condition for 24 hr before 1 nM DHT treatment for 24 hr. qRT-PCR was performed to determine the hTERT mRNA level. As shown in Fig. 7A, at 24 hr treatment, 1 nM DHT could induce hTERT mRNA to ~ 4-fold of control under normoxia condition. The induction became more dramatic (~6-fold) when cells were cultured in hypoxia condition. In addition to LNCaP cells, we also investigated androgen effect on hTERT expression in another androgen-dependent but wild-type AR expressing cell line, LAPC-4. Interestingly, we found that in contrast to up-regulation of hTERT expression in LNCaP cells, DHT actually inhibits hTERT mRNA expression in LAPC-4 cells (in first annual report). We then compared the DHT effect on hTERT expression in this cell line under normoxia vs. hypoxia condition. As shown in Fig. 7B, 1 nM DHT inhibited hTERT mRNA expression to ~50% of control under normoxia condition. However, DHT actually up-regulated hTERT mRNA level to ~ 3 folds of vehicle treated control when LAPC-4 cells were cultured in hypoxia condition. The opposite effect of androgen on hTERT expression indicates that under hypoxia condition which is much similar to tumor environment, there might be some molecules or pathways that could facilitate wild-type AR to activate hTERT expression.

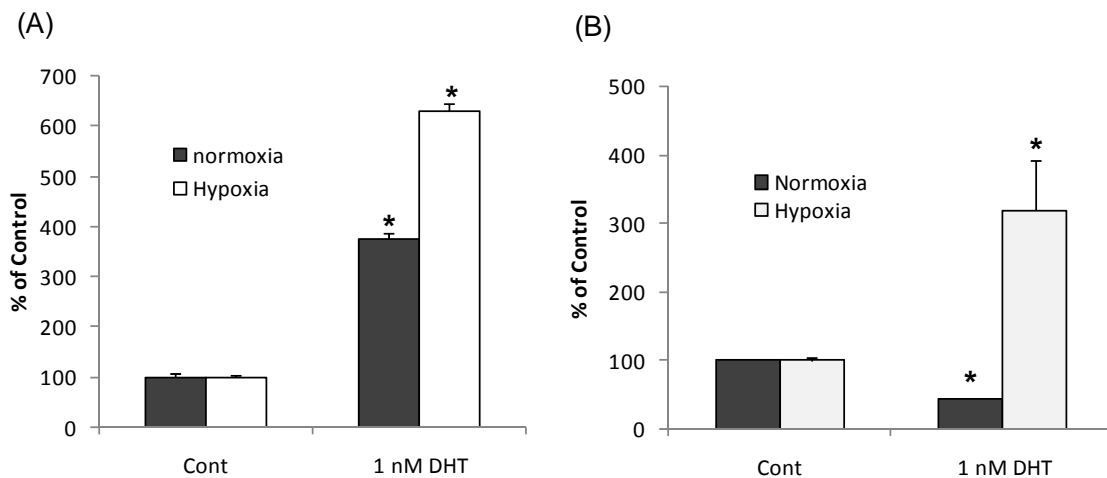


Figure 7. Androgen-AR regulation of hTERT. (A & B) qRT-PCR analysis of hTERT mRNA level in LNCaP (A) and LAPC-4 (B) cells treated with 1 nM DHT for 24 hr under normoxia condition and hypoxia conditions. Data are presented as % of ethanol-treated control cells cultured in charcoal-stripped fetal bovine serum. Bars represent SEM. *, $P < 0.01$ compared to control.

Experiment 3. Selenium inhibits hTERT expression under hypoxia condition in both LNCaP and LAPC-4 cells.

We next examined the effect of MSA on hTERT expression under hypoxia condition in LNCaP and LAPC-4 cells. As shown in Fig. 8A, in LNCaP cells, DHT could induce hTERT mRNA expression to ~ 6 folds; this induction was almost blocked by MSA treatment. We observed the same result in LAPC-4 cells that MSA could also inhibit DHT induced hTERT expression (Fig. 8B). Therefore, our data indicate that MSA has the ability to repress hTERT expression regardless of the culture condition.

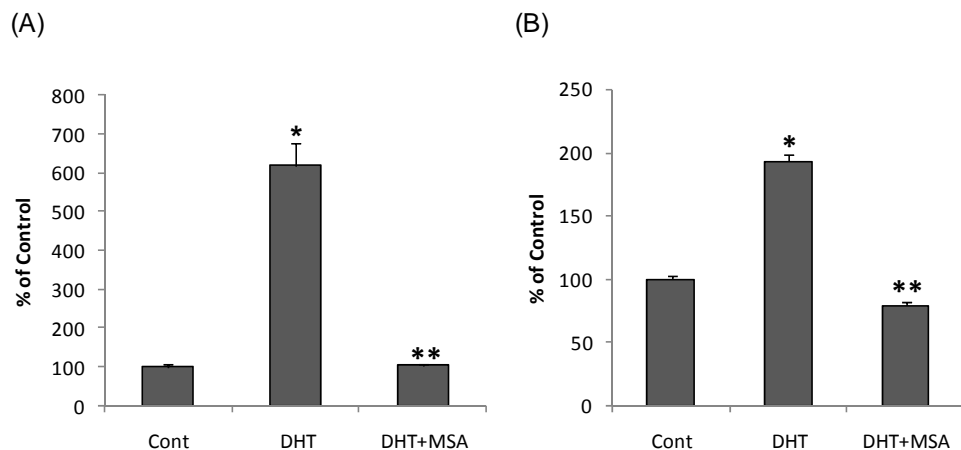


Figure 8. MSA suppression of hTERT expression. LNCaP (A) and LAPC-4 (B) cells were treated with ethanol, 1nM DHT or 1 nM DHT plus 10 μ M MSA for 24 hr. Changes in hTERT mRNA was determined by qRT-PCR. Data are presented as % of ethanol-treated control cells. *, $P < 0.01$, significantly different from control cells. **, $P < 0.01$, significantly different from DHT-treated cells.

We also investigate the effect of bicalutamide in combination with MSA on hTERT expression under hypoxia condition. As shown in Fig. 9, in LNCaP cells we observed the similar combinatorial effect on hTERT suppression as in normoxia culture condition.

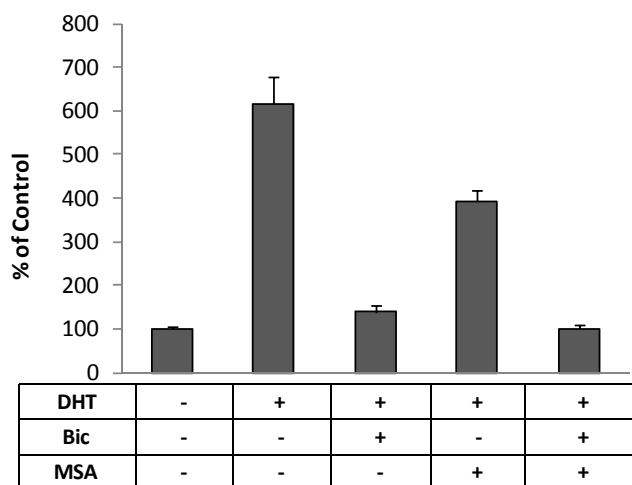


Fig. 9. Combinatorial effect of bicalutamide and MSA on hTERT expression in LNCaP cells cultured under hypoxia condition.

C. KEY RESEARCH ACCOMPLISHMENTS:

- Bicalutamide and selenium combination has greater apoptosis induction in AR-positive cells rather than AR-negative cells compared to single agents.
- Bicalutamide and selenium combination has greater suppression on AR signaling, thus leading to inhibition of telomerase activity.
- Selenium reduces AR recruitment to hTERT promoter region.
- In normoxia and hypoxia conditions, DHT has different effect on hTERT mRNA expression in LAPC-4 cells expressing wild-type AR responses.
- Selenium inhibits hTERT expression under both normoxia and hypoxia condition.

D. REPORTABLE OUTCOMES:

- **Publication(s):**
Liu S, Qi Y, Ge Y, Duplessis T, Rowan BG, Ip C, Cheng H, Rennie PS, Horikawa I, Lustig A, Yu Q, Zhang H, and Dong Y. Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment. Mol Cancer Ther 2010. (*Accepted*)
- **Presentation(s):**
 101st American Association for Cancer Research (AACR) Annual Meeting, 2010 April, "Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment" (*poster presentation*)

E. CONCLUSIONS:

The result from our current study further suggests that hTERT, as a novel target of AR signaling, plays an important role in mediating bicalutamide/selenium effect in human prostate cancer cells. Further continuation of this study will provide a justification for a mechanism-driven strategy in using selenium, in combination with anti-androgens, to control prostate cancer development and progression. We also elucidated that the repression of hTERT by selenium is through inhibition of AR recruitment to hTERT promoter region, thus down-regulation of hTERT transcription. In addition, we demonstrated that wild-type and mutant AR have differential response to androgen; wild-type AR has different response to androgen treatment under normoxia and hypoxia condition. Selenium either alone or combined with bicalutamide

could inhibit hTERT expression in hypoxia condition. Our current finding not only brings a new direction to this project but also has great clinical implications.

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APPENDICES

Telomerase as an important target of androgen-signaling blockade for prostate cancer treatment

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As the mainstay treatment for advanced prostate cancer, androgen-deprivation therapy (ADT) targets the action of androgen receptor (AR) by reducing androgen level and/or by the administration of anti-androgen that competes with androgens for binding to AR. Albeit effective in extending survival, ADT is associated with dose-limiting toxicity and the development of castration-resistant prostate cancer (CRPC) after prolonged use. Since CRPC is generally lethal and incurable, developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. Continuous AR signaling constitutes one major mechanism underlying the development of CRPC. The present study showed that methylseleninic acid (MSA), an agent that effectively reduces AR abundance, could enhance the cancer-killing efficacy of the anti-androgen bicalutamide in both androgen-dependent and castration-resistant prostate cancer cells. We found that combination of MSA and bicalutamide produced a robust downregulation of prostate-specific antigen and a recently identified AR target, telomerase and its catalytic subunit, telomere reverse transcriptase (hTERT). The downregulation of hTERT occurs mainly at the transcriptional level, through reducing AR occupancy of the hTERT promoter. Furthermore, apoptosis induction by the two agents is significantly mitigated by restoration of hTERT. Our findings thus indicate that MSA in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of ADT. Given the critical role of hTERT/telomerase downregulation in mediating the combination effect and the fact that hTERT/telomerase could be measured in blood and urine, hTERT/telomerase could serve as an ideal tumor-specific biomarker to monitor the efficacy of the combination therapy non-invasively. (Supported by the Department of Defense Prostate Cancer Training grant No. W81XWH-08-1-0291 (SL); the National Cancer Institute grant No. K01 CA114252 (YD); the American Cancer Society grant No. RSG-07-218-01-TBE (YD))